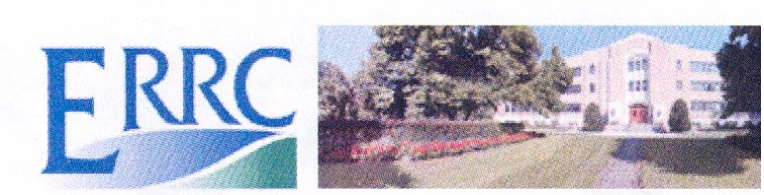

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Title: Detection of Pathogens in Fruits and Vegetables

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DETECTION OF PATHOGENS IN FRUITS AND VEGETABLES

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During the past two decades, human illnesses linked to the consumption of fresh produce, sprouts, and fruit juices contaminated with pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, *Shigella* spp., *Cryptosporidium parvum* and hepatitis A have increased. Factors contributing to the increase include changes in technologies and practices used by the produce industry, including the use of manure instead of chemical fertilizers and the use of untreated water for irrigation, as well as changes in consumption patterns. Contamination can occur in the field, during harvesting due to human handling or harvesting equipment, during processing and shipping, or in the home. Since the type and level of microflora on fresh produce varies considerably, there are currently no microbiological standards for fruits and vegetables. Studies on the sources, levels, and prevalence of different pathogens found in produce are needed to develop effective control strategies. To conduct these studies and to ensure a safe and wholesome food supply, sensitive, reliable, and specific assays for pathogen detection are needed.

The simplest approach for conceptualizing detection technologies and their feasibility is to categorize them into three groups. Cultural-based (conventional) methods involve enrichment for the target organism in liquid medium, plating onto selective agars, and confirmation of the isolate using a series of biochemical and other tests. Since conventional methods are time consuming and may require several days before results are known, they may not be applicable to testing of produce, in particular, minimally processed fruits and vegetables that have a short shelf life. Immunological assays rely on the binding of a monoclonal or polyclonal antibody to a cellular target and include the Enzyme-Linked ImmunoSorbent Assay (ELISA), immunochromatographic assays, the Antibody-Direct Epifluorescent Filter Technique (Ab-DEFT), and others. The sensitivity and specificity of immunoassays depend on the quality of the antibody used. Genetic-based methods, including the polymerase chain reaction (PCR) and DNA hybridization assays rely on binding of segments of nucleic acids to bacterial DNA targets. The rapid detection of pathogens is critical for ensuring safety to consumers. A "rapid method" is a term that describes a test that can provide results in relatively short period of time. Rapid tests include miniaturized biochemical kits, antibody- or nucleic acid- based kits, or conventional methods that are modified to provide results more quickly. Rapid methods can be used for isolation, identification, detection, or characterization of pathogens. The methods used for testing produce are generally similar to those used for testing other foods such as meat, poultry, or milk; however, differences in food matrix components and in the background microflora of produce compared to other types of foods should be considered when interpreting results.

The sensitivity and accuracy of results of food testing are dependent on a number of factors including the type of food being tested, the method of sample collection and preparation, and the size and number of samples collected. Certain types of produce may contain compounds that inhibit the growth of particular bacteria. For example, raw carrots inhibited the growth of *L. monocytogenes*, and tests failed to detect the organism in carrot samples inoculated with low levels of the pathogen (Beuchat and Brackett, 1990; Shearer et al., 2001). The growth of *E. coli* O157:H7 was inhibited by alfalfa sprouts (Shearer et al.,

2001). Methods for sample collection and preparation include swabbing, rinsing, or use of a blender or a Stomacher apparatus to dislodge bacteria from the food or other type of sample. Surface rinsing of produce may not be appropriate for removal of bacteria that may be internalized (Solomon et al., 2002; Buchanan et al., 1999) or that are firmly attached or are found in biofilms (Fig.1; Fett, 2000). In addition, because pathogens may be present at very low levels and not evenly distributed in samples such as seeds, the sample size needs to be sufficiently large to ensure detection of target pathogens.

Fig. 1. Scanning electron micrograph of a native biofilm on an alfalfa sprout hypocotyl (Fett, 2000).

Other factors to consider when developing methodologies for detection of pathogens in fruits and vegetables include the selection of the appropriate enrichment media and detection method. The type and number of background organisms, and whether the target organisms were injured due to the food environment or during processing and storage are factors to consider in the selection of the enrichment medium (Liao and Shollenberger, 2003). The presence of fermented metabolites from the native microflora and/or of components of the material being tested such as components from alfalfa seeds can hinder the repair of injured target organisms (Liao and Fett, 2003). The use of pre-enrichment step increased the frequency of isolation of *Salmonella* (Liao and Fett, 2003). The application of immunomagnetic separation for removal of PCR-inhibitory components or of background microflora present in enrichments increased the ability to detect pathogens by the PCR or by plating (Hara-Kudo, 2000; Liao and Shollenberger, 2003). Fratamico and Bagi (2002) and Shearer et al. (2001) found that the PCR was more sensitive and allowed detection of pathogens more rapidly than culture or immunologic assays.

Previous research conducted at the ERRC (Riordan et al. 2001) demonstrated that constituents of the orchard environment, including fecal matter, soil, irrigation and surface water, and windblown dust are potential sources of *E. coli* O157:H7 contamination of fruit. However, further research is needed before appropriate interventions can be applied to reduce the risk of contamination. To address this research need, a study was designed to determine the presence of *E. coli* O157:H7 in a pasture and if proximity of the pasture to an orchard can result in spread of this organism to the soil and fruit. A protocol was designed to detect coliforms, generic *E. coli*, and *E. coli* O157:H7 in apple, soil, and fecal samples. Apple and soil samples were collected from two independent orchards, orchard A, next to a pasture accessible to wildlife, and orchard B, away from a pasture and inaccessible to wildlife. Within orchard A, apple and soil samples were collected from two different locations, A1, next to the pasture (22 ft from pasture fence), and A2, the interior of orchard (440 ft from the pasture fence). A total of 36 apples were collected from 3 trees (12 per tree) per site, and from 1 soil sample (ca. 100 gm) next to every tree. A total of 3 soil and 3 fecal samples were collected from the pasture from a location next to the fence, opposite to sampled trees from orchard A1. Samples were packed in individual polyethylene bags, and transported to the

laboratory overnight. All samples were collected during the months of June, July, August, and September.

Triplicate composites of six pieces of fruit each were blended with an equal volume of sterile 0.1% peptone water (PW). In addition, the stem, core, and calyx of fruit samples collected were tested for the presence of internalized bacteria. Triplicate sets of 6 stem, 6 core, or 6 calyx portions were individually blended in PW and were filtered. The fruit filtrates were diluted as necessary in PW and plated, with the remainder of the filtrate retained at 4°C. Fruit filtrates were enumerated for total mesophilic aerobic counts on trypticase soy agar (TSA), and for total coliforms and generic *E. coli* on *E. coli*/coliform count Petrifilm plates. Plates were incubated at 35°C and examined at 24 h and 48 h for the presence of coliforms (red colonies with gas) and generic *E. coli* (blue colonies with gas). Filtrates displaying these types of colonies on the Petrifilm plates were subjected to enrichment to determine if the *E. coli* were serotype O157:H7.

Following enrichment of the fruit filtrates, soil, and fecal samples in Gram Negative broth, 1ml of the cultures was subjected to immunomagnetic separation using Dynal anti-O157 magnetic beads (Dynal, Inc., Lake Success, NY) according to the manufacturer protocol. The beads-bacteria complexes were plated onto Sorbitol MacConkey agar (SMAC) and onto SMAC supplemented with 0.05-mg/l cefixime and 2.5 mg/l potassium tellurite (CT-SMAC), then were incubated for 24 h at 35°C. Presumptive *E. coli* O157:H7 colonies, which appeared colorless on CT-SMAC, were transferred to slants of TSA supplemented with 0.6 % yeast extract and incubated overnight at 35°C. Colonies were tested for the O157 antigen with the RIM *E. coli* O157:H7 Latex test (Remel, Lenexa, KS). Positive isolates were subjected to PCR and pulsed field gel electrophoresis (PFGE). The multiplex PCR was performed as described previously (Fratamico et al., 2000). PFGE was performed to test the relatedness of all *E. coli* isolates including the *E. coli* O157:H7 isolates, using the standard protocol used by the Food Safety Inspection Service (Cook, 1998). BioNumerics software (Applied Maths, Austin, TX) was used for caldistic analysis.

Overall, zero, 33, 33, and 100% of fruit samples collected from orchard A (locations A1 and A2) during the months of June, July, August, and September, respectively, were positive for generic *E. coli*. *E. coli* was not detected in any of the apple samples collected from orchard B during this study. *E. coli* O157:H7 was never isolated from any of the apple samples tested from both orchards. Generic *E. coli* were isolated from 100 % of the calyx and stem sections of the apple samples collected from orchard A, locations A1 and A2, during the month of September. Although, *E. coli* was not detected in the core (internalized cells) of the apple, coliforms were found to be internalized in 55, 22, and 33% of the apple samples collected from orchard A, locations A1 and A2, and orchard B, respectively. Soil samples collected from orchard A, locations A1 and A2, and orchard B, and from the pasture showed the presence of generic *E. coli* in 33, 25, 33, and 67% of the samples, respectively. All fecal samples collected from the pasture area tested positive for generic *E. coli*, and in addition, *E. coli* O157:H7 was also isolated from several of the samples. Furthermore, a number of soil samples collected from orchard A, in both locations A1 and A2, tested positive for *E. coli* O157:H7. The multiplex PCR was performed on 6 presumptive *E. coli* O157:H7 isolates originating from soil and fecal samples. Results showed that all of the *E. coli* O157:H7 isolates possessed the $fliC_{h7}$, stx_1, *eae*, and $hlyo_{33}$ genes confirming that the isolates were *E. coli* O157:H7 (Fig. 2). In addition, the 6 *E. coli* O157:H7 isolates were identical by PFGE and a number of sets of non-O157:H7 *E. coli* isolates obtained from fecal, soil, and fruit samples were indistinguishable by *Xba*I PFGE, indicating that *E. coli* can spread throughout the farm/orchard environment.

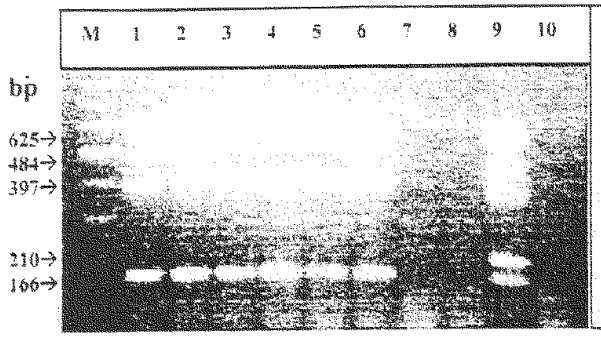

Fig. 2. Ethidium bromide-stained agarose gel showing PCR products obtained following amplification of DNA from *E. coli* O157:H7 isolates from soil (lanes 1 and 2) and from fecal samples (lanes 3-6). Lanes 7 and 8, PCR using DNA from generic *E. coli* strains; lane 9, PCR using DNA from a control strain that harbors both stx_1 and stx_2 genes; lane 10, no template control; M, DNA size markers.

Results of this study showed that use of a combination of a cultural method for isolation, genetic and immunologic methods for confirmation, and typing methods to determine relatedness of isolates can provide valuable information on the presence and source of *E. coli* in the environment and in the food. The study confirms that close proximity of an orchard to a pasture increases the risk of *E. coli* prevalence on apples, although none of the isolates from the apples were *E. coli* O157:H7. Thus, it is advisable that orchards are located away from pastures, and fences should be erected to keep out wild and domestic animals. Due to the potential of contamination of fruit by organisms in the orchard soil, dropped apples should not be included in the production of apple cider.

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